



## Genetic determinants of the ankle-brachial index: A meta-analysis of a cardiovascular candidate gene 50K SNP panel in the candidate gene association resource (CARE) consortium

Christina L. Wassel<sup>a,1</sup>, Claudia Lamina<sup>b,1</sup>, Vijay Nambi<sup>c,1</sup>, Stefan Coassin<sup>b,1</sup>, Kenneth J. Mukamal<sup>d,1</sup>, Santhi K. Ganesh<sup>e</sup>, David R. Jacobs Jr.<sup>f</sup>, Nora Franceschini<sup>g</sup>, George J. Papanicolaou<sup>h</sup>, Quince Gibson<sup>i</sup>, Lisa R. Yanek<sup>j</sup>, Pim van der Harst<sup>k</sup>, Jane F. Ferguson<sup>l</sup>, Dana C. Crawford<sup>m</sup>, Lindsay L. Waite<sup>n</sup>, Matthew A. Allison<sup>a</sup>, Michael H. Criqui<sup>a</sup>, Mary M. McDermott<sup>o</sup>, Reena Mehra<sup>p</sup>, L. Adrienne Cupples<sup>q</sup>, Shih-Jen Hwang<sup>r</sup>, Susan Redline<sup>s</sup>, Robert C. Kaplan<sup>t</sup>, Gerardo Heiss<sup>g</sup>, Jerome I. Rotter<sup>u</sup>, Eric Boerwinkle<sup>v</sup>, Herman A. Taylor<sup>w</sup>, Luis H. Eraso<sup>x</sup>, Margot Haun<sup>b</sup>, Mingyao Li<sup>y</sup>, Christa Meisinger<sup>z</sup>, Jeffrey R. O'Connell<sup>i</sup>, Alan R. Shuldiner<sup>i,A</sup>, Anne Tybjaerg-Hansen<sup>B</sup>, Ruth Frikke-Schmidt<sup>B</sup>, Barbara Kollerits<sup>b</sup>, Barbara Rantner<sup>b,C</sup>, Benjamin Dieplinger<sup>D</sup>, Marietta Stadler<sup>E</sup>, Thomas Mueller<sup>D</sup>, Meinhard Haltmayer<sup>D</sup>, Peter Klein-Weigel<sup>F</sup>, Monika Summerer<sup>b</sup>, H.-Erich Wichmann<sup>G,H,I</sup>, Folkert W. Asselbergs<sup>J,K,L</sup>, Gerjan Navis<sup>M</sup>, Irene Mateo Leach<sup>k</sup>, Kristin Brown-Gentry<sup>N</sup>, Robert Goodloe<sup>N</sup>, Themistocles L. Assimes<sup>O</sup>, Diane M. Becker<sup>j</sup>, John P. Cooke<sup>O</sup>, Devin M. Absher<sup>n</sup>, Jeffrey W. Olin<sup>P</sup>, Braxton D. Mitchell<sup>i</sup>, Muredach P. Reilly<sup>l</sup>, Emile R. Mohler III<sup>Q,1</sup>, Kari E. North<sup>R,1</sup>, Alexander P. Reiner<sup>S,1</sup>, Florian Kronenberg<sup>b,1</sup>, Joanne M. Murabito<sup>T,1,\*</sup>

<sup>a</sup> University of California San Diego, Department of Family and Preventive Medicine, Division of Preventive Medicine, La Jolla, CA, USA

<sup>b</sup> Division of Genetic Epidemiology, Department of Medical Genetics, Molecular and Clinical Pharmacology, Innsbruck Medical University, Innsbruck, Austria

<sup>c</sup> Baylor College of Medicine, Department of Medicine, Section in Cardiology, Houston, TX, USA

<sup>d</sup> Division of General Medicine & Primary Care, Beth Israel Deaconess Medical Center, Boston, MA, USA

<sup>e</sup> Division of Cardiovascular Medicine, Department of Internal Medicine, University of Michigan Health Care System, Ann Arbor, MI, USA

<sup>f</sup> Department of Epidemiology and Community Health, School of Public Health, University of Minnesota, Minneapolis, MN, USA

<sup>g</sup> University of North Carolina, Department of Epidemiology, Gillings Global School of Public Health, Chapel Hill, NC, USA

<sup>h</sup> Division of Cardiovascular Sciences, National Heart, Lung and Blood Institute, Bethesda, MD, USA

<sup>i</sup> Division of Endocrinology, Department of Medicine, University of Maryland School of Medicine, Baltimore, MD, USA

<sup>j</sup> Johns Hopkins University School of Medicine, Division of General Internal Medicine, Baltimore, MD, USA

<sup>k</sup> Department of Cardiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

<sup>l</sup> Perelman School of Medicine at the University of Pennsylvania, Cardiovascular Institute, Philadelphia, PA, USA

<sup>m</sup> Department of Molecular Physiology and Biophysics, The Center for Human Genetics Research, Vanderbilt University, Nashville, TN, USA

<sup>n</sup> HudsonAlpha Institute for Biotechnology, Huntsville, AL, USA

<sup>o</sup> Northwestern University Feinberg School of Medicine, Department of Medicine, Chicago, IL, USA

<sup>p</sup> Case Western Reserve University, University Hospitals Case Medical Center, Cleveland, OH, USA

<sup>q</sup> Boston University School of Public Health, Department of Biostatistics, Boston, MA, USA

<sup>r</sup> The Framingham Heart Study, Center for Population Study, National Heart Lung and Blood Institute, Framingham, MA, USA

<sup>s</sup> Brigham and Women's Hospital, Division of Sleep Medicine, Harvard Medical School, Boston, MA, USA

<sup>t</sup> Albert Einstein College of Medicine, Department of Epidemiology and Population Health, Bronx, NY, USA

<sup>u</sup> Medical Genetics Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA

<sup>v</sup> University of Texas Health Science Center at Houston, Department of Epidemiology, Human Genetics and Environmental Sciences, Houston, TX, USA

<sup>w</sup> University of Mississippi Medical Center AND Jackson State University, Jackson, MS, USA

<sup>x</sup> Jefferson Vascular Center, Thomas Jefferson University Hospital, Philadelphia, PA, USA

<sup>y</sup> Perelman School of Medicine at the University of Pennsylvania, Center for Clinical Epidemiology and Biostatistics, Philadelphia, PA, USA

<sup>z</sup> Institute of Epidemiology II, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany

<sup>A</sup> Geriatric Research and Education Clinical Center, Veterans Administration Medical Center, Baltimore, MD, USA

<sup>B</sup> Department Clin. Biochem., Rigshospitalet, Copenhagen University Hospitals, Copenhagen, Denmark

<sup>C</sup> Department of Vascular Surgery, Innsbruck Medical University, Innsbruck, Austria

\* Corresponding author at: 73 Mt. Wayte Avenue, Framingham, MA 01702, USA. Tel.: +1 508 935 3461; fax: +1 508 626 1262.  
E-mail address: [Murabito@bu.edu](mailto:Murabito@bu.edu) (J.M. Murabito).

<sup>1</sup> Equal work was done by these authors for this paper.

<sup>D</sup> Department of Laboratory Medicine, Konventhospital Barmherzige Brüder Linz, Linz, Austria

<sup>E</sup> Hietzing Hospital, 3rd Medical Department of Metabolic Diseases and Nephrology, Vienna, Austria

<sup>F</sup> Klinik für Innere Medizin – Schwerpunkt Angiologie, DRK-Kliniken Berlin Mitte, Berlin, Germany

<sup>G</sup> Institute of Epidemiology I, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany

<sup>H</sup> Institute of Medical Informatics, Biometry and Epidemiology, Ludwig-Maximilians-Universität, Munich, Germany

<sup>I</sup> Klinikum Grosshadern, Munich, Germany

<sup>J</sup> Department of Cardiology, Division Heart and Lungs, University Medical Center Utrecht, Utrecht, The Netherlands

<sup>K</sup> Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands

<sup>L</sup> Department of Medical Genetics, Biomedical Genetics, University Medical Center, Utrecht, The Netherlands

<sup>M</sup> Department of Internal Medicine, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

<sup>N</sup> The Center for Human Genetics Research, Vanderbilt University, Nashville, TN, USA

<sup>O</sup> Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA

<sup>P</sup> Zena and Michael A. Wiener Cardiovascular Institute & Marie-José and Henry R. Kravis Center for Cardiovascular Health, Mount Sinai School of Medicine, New York, NY, USA

<sup>Q</sup> Perelman School of Medicine at the University of Pennsylvania, Cardiovascular Division, Vascular Medicine Section, Philadelphia, PA, USA

<sup>R</sup> Department of Epidemiology and Carolina Center for Genome Sciences, The University of North Carolina, Chapel Hill, NC, USA

<sup>S</sup> Department of Epidemiology, University of Washington School of Public Health, Seattle, WA, USA

<sup>T</sup> Boston University School of Medicine, Department of Medicine, Section of General Internal Medicine; NHLBI's Framingham Heart Study, Boston, MA, USA

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## ABSTRACT

**Background:** Candidate gene association studies for peripheral artery disease (PAD), including subclinical disease assessed with the ankle-brachial index (ABI), have been limited by the modest number of genes examined. We conducted a two stage meta-analysis of ~50,000 SNPs across ~2100 candidate genes to identify genetic variants for ABI.

**Methods and results:** We studied subjects of European ancestry from 8 studies ( $n = 21,547$ , 55% women, mean age 44–73 years) and African American ancestry from 5 studies ( $n = 7267$ , 60% women, mean age 41–73 years) involved in the candidate gene association resource (CARE) consortium. In each ethnic group, additive genetic models were used (with each additional copy of the minor allele corresponding to the given beta) to test each SNP for association with continuous ABI (excluding  $ABI > 1.40$ ) and PAD (defined as  $ABI < 0.90$ ) using linear or logistic regression with adjustment for known PAD risk factors and population stratification. We then conducted a fixed-effects inverse-variance weighted meta-analysis considering a  $p < 2 \times 10^{-6}$  to denote statistical significance.

**Results:** In the European ancestry discovery meta-analyses, rs2171209 in *SYTL3* ( $\beta = -0.007$ ,  $p = 6.02 \times 10^{-7}$ ) and rs290481 in *TCF7L2* ( $\beta = -0.008$ ,  $p = 7.01 \times 10^{-7}$ ) were significantly associated with ABI. None of the SNP associations for PAD were significant, though a SNP in *CYP2B6* ( $p = 4.99 \times 10^{-5}$ ) was among the strongest associations. These 3 genes are linked to key PAD risk factors (lipoprotein(a), type 2 diabetes, and smoking behavior, respectively). We sought replication in 6 population-based and 3 clinical samples ( $n = 15,440$ ) for rs290481 and rs2171209. However, in the replication stage (rs2171209,  $p = 0.75$ ; rs290481,  $p = 0.19$ ) and in the combined discovery and replication analysis the SNP-ABI associations were no longer significant (rs2171209,  $p = 1.14 \times 10^{-3}$ ; rs290481,  $p = 8.88 \times 10^{-5}$ ). In African Americans, none of the SNP associations for ABI or PAD achieved an experiment-wide level of significance.

**Conclusions:** Genetic determinants of ABI and PAD remain elusive. Follow-up of these preliminary findings may uncover important biology given the known gene-risk factor associations. New and more powerful approaches to PAD gene discovery are warranted.

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## 1. Introduction

Peripheral artery disease (PAD) is associated with an increased risk for incident cardiovascular disease events and mortality [1,2]. In the reduction of atherothrombosis for continued health (REACH) registry, almost two-thirds of the individuals with PAD had concomitant clinically evident atherosclerotic disease in the cerebrovascular or coronary artery disease (CAD) territories whereas only one-quarter of the individuals with coronary disease had clinically evident atherosclerotic involvement of other arterial beds [3]. While PAD and CAD share many common risk factors, cigarette smoking and type 2 diabetes are stronger risk factors for PAD than for coronary artery disease [4]. The variable distribution of the burden of atherosclerosis across vascular beds among subjects at risk suggests that other factors exist, including possibly genetic factors, that may contribute to the predilection of atherosclerosis to develop in a given anatomic location. Currently, little is known about the genetic susceptibility to PAD but familial aggregation of PAD and heritability estimates suggest a significant genetic contribution [5–10].

The ankle brachial index (ABI) is an easy and reliable diagnostic test used to detect symptomatic as well as asymptomatic PAD [11]. A genome-wide linkage scan for ABI identified several potential

candidate genes under six linkage signals in pathways of inflammation, coagulation, blood pressure regulation, and lipid metabolism [8]. A recent large genome-wide association study (GWAS) meta-analysis of European descent participants found variants in the 9p21 locus significantly associated with ABI [12]. However, there have been few candidate gene association studies for PAD, most of which have been limited by small sample size, modest number of genes examined, and lack of robust independent replication of initial findings [13]. We conducted a two stage large scale candidate gene association study of the ~2100 candidate genes included in a cardiovascular gene-centric 50K single nucleotide polymorphism (SNP) array [14] within the candidate gene association resource (CARE) consortium that included 21,547 individuals of European ancestry (1190 with  $ABI < 0.9$ ) from eight cohorts and 7267 African American individuals (594 with  $ABI < 0.90$ ) from six cohorts. In the second stage of the investigation, we sought to replicate our significant associations among individuals of European ancestry in 13,524 individuals from population-based cohort studies and 1916 individuals from clinically based studies. We hypothesized that this approach would lead to the identification of novel genetic variants associated with ABI and PAD (as defined by an  $ABI < 0.90$ ). Furthermore, we hypothesized that some variants may influence both PAD risk factors and PAD itself, as has been observed

in genome-wide studies of lipids and coronary artery disease [15,16].

## 2. Methods

### 2.1. Discovery studies: CARE consortium and additional studies

The CARE consortium (<http://public.nhlbi.nih.gov/GeneticsGenomics/home/care.aspx>) was funded by the National Heart Lung and Blood Institute (NHLBI) in 2006 to explore the association of a custom cardiovascular gene centric SNP array [14] with a broad set of cardiovascular, metabolic, and inflammatory phenotypes collected across nine longitudinal cohort studies [17]. The following CARE consortium studies contributed data to the present analysis (Tables 1A and 1B): atherosclerosis risk in communities study (ARIC,  $n=9031$  European Americans,  $n=2853$  African Americans), the Cleveland family study (CFS,  $n=275$  European Americans,  $n=365$  African Americans), the cardiovascular health study (CHS,  $n=3826$  European Americans,  $n=722$  African Americans), the Framingham heart study (FHS,  $n=2701$  European Americans), the Jackson heart study (JHS,  $n=1734$  African Americans), and the multi-ethnic study of atherosclerosis (MESA,  $n=2280$  European Americans,  $n=1593$  African Americans). The Amish study ( $n=1008$ ), the cooperative research in the region of Augsburg (KORA F3,  $n=1807$ ), and the Penn diabetes heart study (PDHS,  $n=622$ ) cohort all used the same cardiovascular gene centric SNP array and joined the discovery stage analyses conducted in European Americans.

Description of each study is provided in the [Supplementary Materials](#). For all studies, each participant self-identified as either White (European, European American) or African American and provided written informed consent. The Institutional Review Board at the parent institution for each respective study approved the study protocols.

The characteristics of the discovery study samples at the time of ABI measurement are presented in Tables 1A and 1B by ethnic group. More than half were women and the mean age ranged from 44 years (CFS) to 73 years (CHS) in samples of European ancestry, and from 41 years (CFS) to 73 years (CHS) in African Americans. The mean ABI was 1.10 and the prevalence of PAD (ABI < 0.90) varied across studies, ranging from 4% to 12% in European Americans and from 5% to 21% in African Americans. Risk factor burden appeared greater in African Americans, as demonstrated by higher prevalence of hypertension, type 2 diabetes, and obesity compared to European ancestry participants.

### 2.2. ABI phenotypes

The details of the ABI protocol for each study are provided in [Supplementary Materials, Supplementary Methods Table 1](#). For each leg, the systolic blood pressure at each ankle was divided by the systolic blood pressure in the arm. In the ARIC study, ABI was measured in only one leg and one arm chosen at random. The lower of the two ABIs calculated with each leg was used for analyses with the exception of the Amish study which used the average of an individual's two ABIs in the analyses.

We defined two PAD phenotypes for genetic association analyses. First, we used the continuous range of  $ABI \leq 1.40$ . Next, we defined PAD as  $ABI < 0.90$  and conducted a case ( $ABI < 0.9$ )/control comparison ( $ABI \geq 0.90$  and  $< 1.40$ ) analysis. Participants with an  $ABI > 1.40$  were excluded as these subjects likely had medial artery calcification and therefore the artery would not be compressible to allow for determination of pressure in the artery. Excluding participants with  $ABI$  did not truncate the distribution substantially, and  $ABI$  was still normally distributed.

**Table 1A**  
Characteristics of discovery samples at the time of ankle brachial index (ABI) measurement. European and European Americans.<sup>a</sup>

Characteristic mean (SD), or n (%)	ARIC n = 9031	CFS n = 275	CHS n = 3826	FHS <sup>b</sup> n = 2701	MESA n = 2280	Amish study n = 1008	KORA F3 n = 1807	PDHS n = 622
Age (years)	54 ± 6	44 ± 19	73 ± 6	61 ± 9	63 ± 10	44 ± 14	62 ± 11	59 ± 9
Women (%)	4871 (54%)	145 (53%)	2152 (56%)	1453 (54%)	1196 (52%)	484 (48%)	965 (53%)	429 (69%)
ABI (mean)	1.13 ± 0.13	1.08 ± 0.10	1.06 ± 0.16	1.13 ± 0.12	1.11 ± 0.12	1.10 ± 0.10	1.11 ± 0.13	1.12 ± 0.14
PAD (ABI < 0.9) (%)	334 (4%)	12 (4%)	453 (12%)	100 (4%)	105 (5%)	36 (3.6%)	92 (5%)	40 (6.5%)
Hypertension (%)	2410 (27%)	85 (31%)	2136 (56%)	629 (23%)	882 (39%)	125 (12%)	1046 (58%)	361 (58%)
Diabetes (%)	785 (9%)	34 (12%)	547 (14%)	288 (11%)	141 (6%)	6 (0.6%)	168 (9%)	622 (100%)
Body mass index (kg/m <sup>2</sup> )	27 ± 5	32 ± 9	26 ± 4	28 ± 5	28 ± 5	27 ± 5	28 ± 5	33 ± 6
Ever smoker (%)	5387 (60%)	120 (44%)	2076 (54%)	1595 (59%)	1274 (56%)	239 (24%)	929 (51%)	361 (58%)
Total cholesterol (mg/dL)	215 ± 41	192 ± 42	211 ± 39	200 ± 36	196 ± 36	209 ± 47	222 ± 40	177 ± 38
LDL cholesterol (mg/dL)	137 ± 38	102 ± 30	130 ± 35	119 ± 33	117 ± 30	139 ± 4	130 ± 33	99 ± 30
HDL cholesterol (mg/dL)	51 ± 17	44 ± 12	54 ± 16	54 ± 17	52 ± 16	56 ± 15	59 ± 17	46 ± 13
Triglyceride (mg/dL)	137 ± 93	139 ± 101	144 ± 79	137 ± 89	134 ± 92	69 ± 41	171 ± 133	155 ± 88
Lipid lowering meds (%)	297 (3%)	40 (15%)	73 (2%)	559 (21%)	414 (18%)	32 (2%)	247 (14%)	386 (62%)
Claudication (%)	70 (0.8%)	3 (1%)	79 (2%)	45 (2%)	9 (0.4%)	N/A <sup>c</sup>	67 (3.7%)	0 (0%)
Prevalent CVD (%)	0 (0%)	14 (5%)	879 (23%)	110 (4%)	0 (0%)	28 (2.9%)	227 (13%)	0 (0%)

<sup>a</sup> ARIC = atherosclerosis risk in communities; CFS = Cleveland family study; CHS = cardiovascular health study; FHS = Framingham heart study; MESA = multi-ethnic study of atherosclerosis; KORA F3 = cooperative research in the region of Augsburg; PDHS = Penn diabetes heart study.

<sup>b</sup> Includes both original cohort and offspring cohort.

<sup>c</sup> N/A = not measured or assessed in the study.

**Table 1B**  
Characteristics of discovery samples at the time of ankle brachial index (ABI) measurement. African Americans.<sup>a</sup>

Characteristic mean (SD) or n (%)	ARIC n = 2853	CFS n = 365	CHS n = 722	JHS n = 1734	MESA n = 1593
Age (years)	53 ± 6	41 ± 19	73 ± 6	50 ± 12	62 ± 10
Women (%)	1798 (63%)	208 (57%)	454 (63%)	1051 (61%)	867 (54%)
ABI (mean)	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	1.1 ± 0.1
PAD (ABI < 0.9) (%)	148 (5%)	45 (12%)	153 (21%)	105 (6%)	143 (9%)
Hypertension (%)	1578 (55%)	155 (42%)	520 (72%)	970 (56%)	959 (60%)
Diabetes (%)	543 (19%)	72 (20%)	170 (24%)	251 (14%)	281 (18%)
Body mass index (kg/m <sup>2</sup> )	30 ± 6	33 ± 9	28 ± 6	32 ± 7	30 ± 6
Ever smoker (%)	1500 (53%)	175 (48%)	367 (51%)	544 (31%)	860 (54%)
Total cholesterol (mg/dL)	215 ± 45	183 ± 43	209 ± 39	197 ± 39	189 ± 36
LDL cholesterol (mg/dL)	138 ± 43	97 ± 34	128 ± 36	126 ± 36	116 ± 33
HDL cholesterol (mg/dL)	55 ± 18	44 ± 13	58 ± 16	51 ± 14	52 ± 15
Triglyceride (mg/dL)	113 ± 74	103 ± 63	116 ± 63	106 ± 89	105 ± 71
Lipid lowering meds (%)	39 (1%)	50 (14%)	32 (4%)	154 (9%)	252 (16%)
Claudication (%)	18 (0.6%)	3 (0.8%)	11 (2%)	534 (30%)	10 (0.6%)
Prevalent CVD (%)	0 (0%)	11 (3%)	191 (26%)	98 (6%)	0 (0%)

<sup>a</sup> ARIC = atherosclerosis risk in communities, CFS = Cleveland family study, CHS = cardiovascular heart study, JHS = Jackson heart study, MESA = multi-ethnic study of atherosclerosis.

### 2.3. Genotyping

Genotyping in the CARE cohorts and PDHS was conducted at the Broad Institute using the ITMAT-Broad-CARE (IBC) Illumina iSELECT custom array [14]. The Old Order Amish Study genotyping was also performed using the ITMAT IBC Illumina iSELECT custom array. For KORA F3 (Discovery stage) genotyping with the same array was performed in the Genome Analysis Centre, Helmholtz Zentrum München.

The IBC array was designed to capture genetic variation in loci known or postulated to be associated with cardiovascular disease, metabolic disease and inflammatory diseases [14]. Specifically, a cosmopolitan tagging approach was used to capture genetic diversity across ~2100 candidate genes [14]. Loci were primarily chosen in three groups as follows: (1) 435 loci were chosen areas with a high probability of functional significance, (2) 1349 loci were chosen as having involvement in phenotypes of interest (i.e. cardiovascular disease or cardiovascular disease-related traits such as inflammation, hemostasis, obesity, diabetes) or were well-established loci requiring a number of SNPs for coverage, and (3) 232 lower priority loci were chosen, which also included larger genes [14]. Further details on the IBC array can be found elsewhere [14]. Details of the genotyping and quality control procedures are provided in Supplementary Methods Table 2.

### 2.4. Statistical analysis

For each study, residuals of ABI stratified by gender and race were created from linear regression models and used as phenotypes in the association analysis; results of gender were pooled but all analyses were stratified by race. The ABI residuals were adjusted for age, clinic site for multi-site studies, principal components (participants of European ancestry) or global European ancestry (African American participants), ever smoking, type 2 diabetes, hypertension (>140/90 or use of anti-hypertensive medication), LDL cholesterol, HDL cholesterol, and body mass index (BMI). In each ethnic group, SNPs were modeled additively, and the association of each SNP with ABI was tested using linear regression. The PDHS study did not use diabetes as a covariate as all subjects were diagnosed with type 2 diabetes. For CFS and FHS, linear mixed effects (LME) models were used to account for familial correlations. Multivariable logistic regression was used to test for the association of each SNP with PAD. For CFS and FHS, generalized estimating equations (GEE) were used to account for familial correlations. The covariate adjustment for PAD was the same as used for the ABI phenotype.

A fixed effects meta-analysis with inverse-variance weighting was then conducted in PLINK V 1.0.6 [18] and Stata V 9.0 (College Station, TX) to combine the results for all studies. The association of each additional copy of the minor allele with ABI was quantified by the regression slope ( $\beta$ ), its standard error [SE( $\beta$ )] and the corresponding *p*-value. We calculated a meta-analysis odds ratio (OR) for each of the most significant SNP associations for PAD. The meta-analysis OR represents the increase/decrease in odds of PAD for each additional copy of the minor allele of the SNP. We also tested for heterogeneity of study-specific regression parameters using the Cochran's Q statistic in Stata V9.0, and report the *p*-values for heterogeneity. Associations were considered to be significant on an experiment-wide level at a *p*-value  $\leq 2 \times 10^{-6}$  which was determined based on the estimate of the number of independent tests [19]. SNPs with MAF < 0.01 were excluded.

A gene-based test of association using the meta-analyzed *p*-values at the discovery stage was performed using the program Versatile Gene-Based Association Study (VEGAS) [20]; <http://gump.qimr.edu.au/VEGAS/>). The SNPs are matched to genes using the UCSC Genome Browser hg18 assembly with the gene region defined by  $\pm 50$  kb up- and downstream of the gene. The test is based on the sum of chi-square-statistics and the linkage disequilibrium (LD) of these SNPs is taken into account according to the correlation structure in the HapMap CEU samples. An empirical *p*-value is provided based on all SNPs, as well as based on the SNPs within the top 20% with regard to their *p*-value. Since roughly 2100 genes are covered by the IBC chip, a *p*-value  $< 2.4 \times 10^{-5}$  is considered significant.

### 2.5. Replication

Given that contemporary genetics consortia and results indicate that very large replication samples are needed to successfully replicate SNPs, and that false positives are an ongoing issue in studies such as these, we attempted to replicate only the two SNPs that met experiment-wide significance for ABI in European Americans in an additional 13,524 individuals of European ancestry from six population-based studies (Copenhagen city heart study, *n* = 5182; genetic study of aspirin responsiveness (GeneSTAR), *n* = 618; KORA F3 (independent of KORA F3 participants in the discovery sample), *n* = 1440; KORA F4, *n* = 411; national health and nutrition examination survey (NHANES), *n* = 2358, and prevention of renal and vascular end-stage disease (PREVEND), *n* = 3515) and 1916 individuals of European ancestry from clinically based samples (cardiovascular disease in intermittent claudication (CAVASIC), *n* = 434; genetic determinants of peripheral arterial disease (GenePAD), *n* = 811; and Linz peripheral arterial disease

**Table 2A**Candidate gene SNP associations for ankle-brachial index: discovery meta-analysis,  $p < 10^{-4}$ . European and European Americans,  $n = 21,547$ .

SNP	Chr	Physical position	Nearest gene	Feature	Major/minor allele	MAF <sup>a</sup>	Beta	95% CI	$p$ Value	$p_{het}$ <sup>b</sup>
rs2171209	6	159,103,550	SYTL3	Intron	C/T	0.22	-0.007	-0.010, -0.004	$6.02 \times 10^{-7}$	0.55
rs290481	10	114,913,815	TCF7L2	Intron	C/T	0.17	-0.008	-0.011, -0.005	$7.01 \times 10^{-7}$	0.08
rs11061318	12	130,135,058	GPR133	Missense Ser → Leu	C/T	0.03	-0.016	-0.023, -0.009	$4.46 \times 10^{-6}$	0.91

<sup>a</sup> MAF = minor allele frequency.<sup>b</sup>  $p$ -for-heterogeneity by Cochran's  $Q$ .

(LIPAD),  $n = 671$ ). GeneSTAR provided in silico genotyping (genotyped participants with the same candidate gene chip used in the discovery cohorts) while in the remaining studies genotyped the 2 SNPs de novo using Taqman or Sequenom genotyping platforms. Description of the replication studies, ABI protocol and calculation, and participant characteristics are provided in [Supplementary Methods](#), [Supplementary Method Table 1](#) and [Supplementary Results Table 1](#).

### 2.6. Power and sample size for discovery and replication stages

**Participants of European ancestry:** With a minor allele frequency (MAF) of 0.10, additive SNP modeling, and experiment-wide significance level of  $2 \times 10^{-6}$ , for each additional copy of the risk allele, we have 80% power to detect a beta coefficient for ABI of 0.0092 and an OR for PAD of 1.45 in the discovery stage ( $n = 21,547$ ). For European ancestry individuals in the replication stage for ABI ( $n = 15,440$ ) using the more stringent significance level of  $2 \times 10^{-6}$  we have 80% power to detect a beta coefficient of 0.0106; using a less stringent level (i.e.  $\alpha = 0.025$  based on carrying forward 2 SNPs for replication), we have 80% power to detect a beta coefficient of 0.0058. In the discovery plus replication stage for ABI ( $n = 36,987$ ), we have 80% power to detect a beta coefficient of 0.0065 using  $2 \times 10^{-6}$  as the type 1 error rate.

**Participants of African-American ancestry:** With a minor allele frequency (MAF) of 0.10, additive SNP modeling, and experiment-wide significance level of  $2 \times 10^{-6}$ , for each additional copy of the

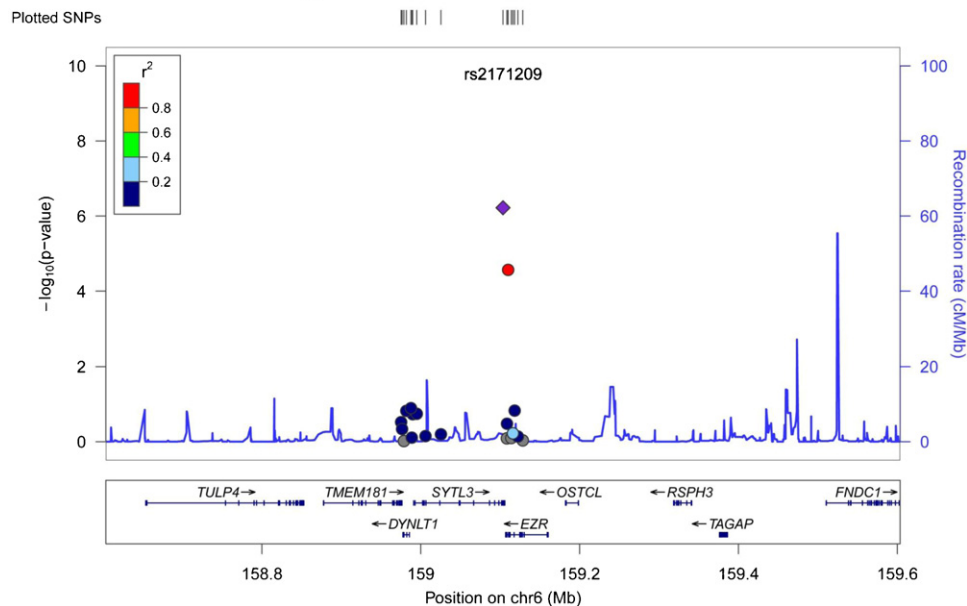
risk allele, we have 80% power to detect a beta coefficient for ABI of 0.0155 and an OR for PAD of 1.67 in the sample of 7267 individuals in the discovery stage. No replication sample was available for this ethnicity.

## 3. Results

### 3.1. European ancestry studies: meta-analysis of ABI and PAD

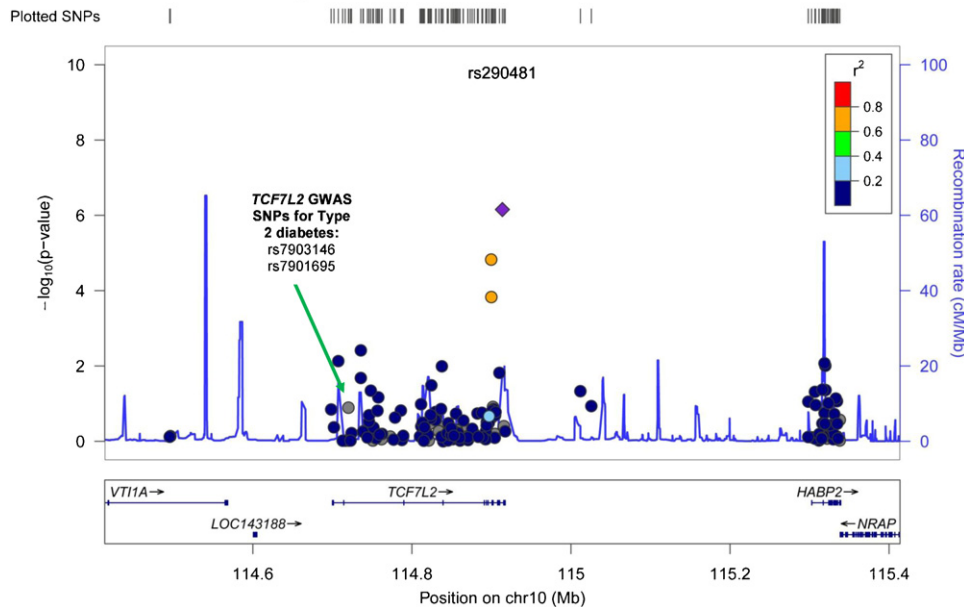
In European ancestry discovery samples, two SNPs were significantly associated with ABI ([Table 2A](#), [Figs. 1 and 2](#)): each additional copy of the minor allele of rs2171209 in SYTL3 was associated with a 0.007 lower ABI (95% CI -0.010, -0.004,  $p = 6.02 \times 10^{-7}$ ,  $p$  for heterogeneity = 0.55) and each additional copy of the minor allele of rs290481 in TCF7L2 was associated with a 0.008 lower ABI (95% CI -0.011, -0.005,  $p = 7.01 \times 10^{-7}$ ,  $p$  for heterogeneity = 0.08). Rs290481 is located in intron 14 within the 3' region of the TCF7L2 gene on chromosome 10 is distinct from a cluster of SNPs in the 5' region of TCF7L2 (represented by rs7903146), previously reported to be associated with type 2 diabetes in genome-wide association studies ( $r^2 = 0.001$  between rs290481 and rs7903146) [21]. Among those of European ancestry, rs2171209 was not significantly associated with the categorical PAD diagnosis made by the ABI threshold of 0.90 – each additional copy of the minor allele was associated with just a 1.09-fold greater odds of PAD (95% CI: 0.97, 1.22,  $p = 0.14$ ); however, rs290481 was associated with PAD (OR = 1.20, 95% CI: 1.06, 1.35,  $p = 0.004$ ), although this

## Regional Association Plot SYTL3



**Fig. 1.** This plot shows the  $p$ -values for rs2171209, as well as for SNPs in the region of rs2171209, with ABI in a meta-analysis of the discovery studies. The x-axis shows chromosomal location in Mb (chromosome 10), as well as genes residing in this region. The y-axis on the left displays the  $-\log_{10}(p\text{-value})$  for each SNP, and the y-axis on the right shows the recombination rate in this region. The top SNP, rs2171209 is represented as a purple diamond, while supporting SNPs and other SNPs in the area are color-coded by linkage disequilibrium with rs2171209 (see  $r^2$  linkage disequilibrium legend on the plot). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

## Regional Association Plot TCF7L2



**Fig. 2.** This plot shows the  $p$ -values for rs290481, as well as for SNPs in the region of rs290481, with ABI in a meta-analysis of the discovery studies. The  $x$ -axis shows chromosomal location in Mb (chromosome 10), as well as genes residing in this region. The  $y$ -axis on the left displays the  $-\log_{10}(p\text{-value})$  for each SNP, and the  $y$ -axis on the right shows the recombination rate in this region. The top SNP, rs290481 is represented as a purple diamond, while supporting SNPs and other SNPs in the area are color-coded by linkage disequilibrium with rs290481 (see  $r^2$  linkage disequilibrium legend on the plot). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

association did not meet the experiment-wide significance level. There was no association between rs290481 and ABI ( $\beta = -0.001$  95% CI:  $-0.006, 0.005, p = 0.80$ ) or rs2171209 and ABI ( $\beta = -0.002$  95% CI  $-0.009, 0.004, p = 0.46$ ) detected in African Americans. One additional SNP was nominally associated with ABI in individuals of European ancestry at  $p < 10^{-4}$  (Table 2A). The most significant SNP associations for ABI in models minimally adjusted for age, sex, and study site only remained similar to the fully adjusted models. When we removed all participants with type 2 diabetes from the analysis of the CARE studies (ARIC, CFS, CHS, FHS and MESA), the beta coefficients were essentially unchanged from those in Table 2A. Similarly, when we removed participants less than 60 years of age from the analysis in the CARE studies, beta coefficients were essentially unchanged from those in Table 2A.

Because rs290481 is located in a gene previously strongly associated with type 2 diabetes, we conducted a test for interaction of this genotype with type 2 diabetes for ABI within the CARE discovery cohorts (ARIC, CFS, CHS, FHS, MESA). The test for interaction was performed on an additive scale using linear regression within each CARE cohort, and then combining the results in the diabetes and no diabetes strata using fixed effect inverse-variance weighting meta-analysis. The magnitude of the effect with ABI was greater in diabetics ( $p$  for interaction  $0.04$ , Supplementary Results Table 2, with each copy of the minor allele in participants with diabetes conferring a lower level of ABI ( $n = 1896, \beta = -0.03, p < 0.001$ ) compared to participants without diabetes ( $n = 16,685, \beta = -0.007, p = 0.007$ ).

The gene-based analysis using VEGAS at the discovery stage did not reveal any different significantly associated genes with continuous ABI from our individual SNP analysis (Supplementary Results Table 3). *SYTL3* and *TCF7L2* were among the most significant genes for both analyses using all SNPs and the top 20% of SNPs (*SYTL3*:  $p(\text{all SNPs}) = 0.00216, p(\text{top20\% of SNPs}) = 0.00007$ ; *TCF7L2*:  $p(\text{all SNPs}) = 0.03589, p(\text{top20\% of SNPs}) = 0.00161$ ).

In the replication stage, the association between ABI and rs2171209 in *SYTL3* was not significant in the population-based ( $\beta = -0.0004, n = 13,510, p = 0.73$ ) and clinically based replication

samples ( $\beta = 0.001, n = 1890, p = 0.82$ ) (Fig. 3). Consequently, in the combined discovery plus replication meta-analysis the association was no longer significant ( $n = 36,947, \beta = -0.003, p = 1.14 \times 10^{-3}$ ) (Fig. 3). rs290481 in *TCF7L2* also failed to replicate in the population-based replication studies ( $\beta = -0.001, n = 13,505, p = 0.38$ ) and in the clinically based replication studies ( $\beta = -0.008, n = 1896, p = 0.20$ ) (Fig. 4). In the combined discovery and replication meta-analysis the association between rs290481 and ABI no longer met experiment-wide significance ( $n = 36,855, \beta = -0.004, p = 8.88 \times 10^{-5}$ ) (Fig. 4).

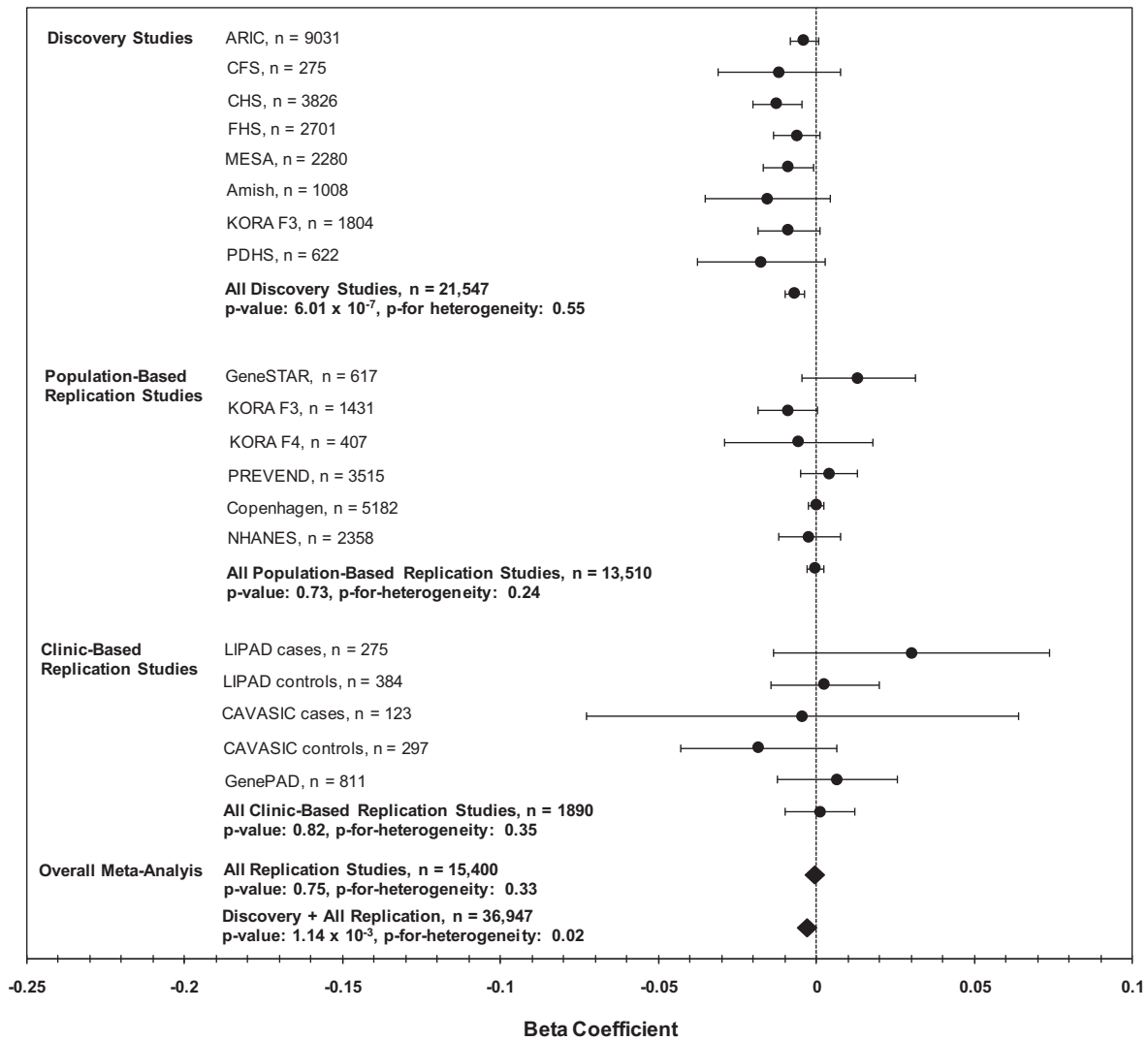
None of the SNP associations in individuals of European ancestry achieved experiment-wide significance for PAD (Table 2B). One of the most significant associations for PAD was in a coding, non-synonymous SNP rs3745274 on chromosome 19 in *CYP2B6* (OR 1.24,  $p = 4.99 \times 10^{-5}$ ).

### 3.2. African-Americans: meta-analysis of ABI and PAD

In African Americans none of the SNP associations with ABI and PAD were statistically significant (Tables 3A and 3B). The strongest association for ABI was rs2243100 on chromosome 17 in *SLC25A11* ( $\beta$  0.011 95% CI 0.006, 0.017,  $p = 5 \times 10^{-5}$ ) and for PAD was rs4987756 on chromosome 18 in *BCL2* (OR 2.99, 95% CI 1.88, 4.76,  $p = 3.78 \times 10^{-6}$ ).

## 4. Discussion

We conducted a large candidate gene association study of ~2100 cardiovascular candidate genes for ABI and PAD in over 21,000 individuals of European ancestry and over 7000 African Americans. In individuals of European ancestry, a SNP in the *TCF7L2* gene (rs290481) and a SNP in the *SYTL3* gene (rs2171209) were significantly associated in the discovery stage with variation in ABI measurements and a suggestive association was identified in a SNP in *CYP2B6* for PAD. These findings are intriguing as the genes are linked to key PAD risk factors. *TCF7L2* is the strongest



**Fig. 3.** This plot shows the association of rs2171209 with ABI for each of the discovery and replication studies, and then results with these studies combined by meta-analysis. The x-axis contains beta coefficients for the association of rs2171209 with ABI in the discovery and replication studies, and the y-axis shows studies or groups of studies. Circles represent the beta coefficient (except for overall replication and discovery + replication meta-analysis results, where beta coefficients are designated by a diamond), and error bars are 95% confidence intervals. *p*-Values for heterogeneity are by Cochran's Q. Study abbreviations are as follows: ARIC = atherosclerosis risk in communities, CFS = Cleveland family study, CHS = cardiovascular health study, FHS = Framingham heart study, MESA = multi-ethnic study of atherosclerosis, KORA F3 and KORA F4 = cooperative research in the region of Augsburg, PDHS = Penn diabetes heart study, GeneSTAR = genetic study of aspirin responsiveness, PREVEND = prevention of renal and vascular end-stage disease, Copenhagen = Copenhagen city heart study, NHANES = national health and nutrition examination survey, LIPAD = Linz peripheral arterial disease, CAVASIC = cardiovascular disease in intermittent claudication, and GenePAD = genetic determinants of peripheral arterial disease.

**Table 2B**  
Candidate gene SNP associations for PAD (ABI < 0.9): discovery meta-analysis, *p* < 10<sup>-4</sup>. European and European Americans, *n* = 20,539.

SNP	Chr	Physical position	Nearest gene	Feature	Major/minor allele	MAF <sup>a</sup>	Odds ratio	95% CI	<i>p</i> Value	<i>p</i> <sub>het</sub> <sup>b</sup>
rs11088283	21	34,745,649	KCNE1	Intron	A/G	0.47	0.85	(0.78, 0.93)	4.88 × 10 <sup>-5</sup>	0.09
rs3745274	19	46,204,681	CYP2B6	Coding, non-synonymous	G/T	0.24	1.24	(1.12, 1.38)	4.99 × 10 <sup>-5</sup>	0.01
rs12428227	13	109,700,293	COL4A1	Intron	A/G	0.17	1.23	(1.10, 1.38)	5.20 × 10 <sup>-5</sup>	0.14
rs17151901	8	10,290,865	MSRA	Intron	C/T	0.02	1.36	(1.09, 1.69)	6.53 × 10 <sup>-5</sup>	0.01

<sup>a</sup> MAF = minor allele frequency.

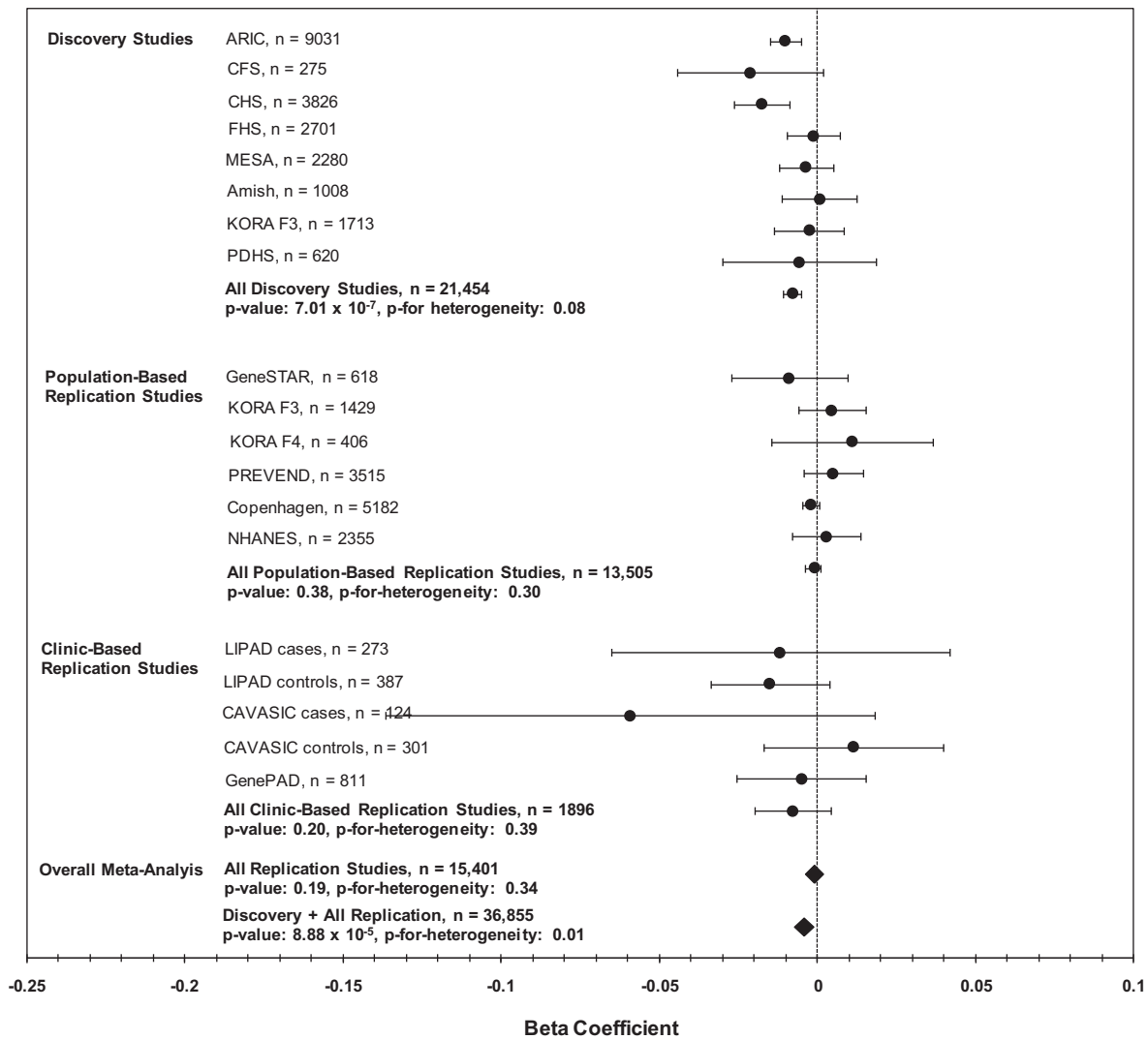
<sup>b</sup> *p*-for-heterogeneity by Cochran's Q.

**Table 3A**  
Candidate gene SNP associations for ankle-brachial index: discovery meta-analysis, *p* < 10<sup>-4</sup>. African Americans, *n* = 7267.

SNP	Chr	Physical position	Nearest gene	Feature	Major/minor allele	MAF <sup>a</sup>	Beta	95% CI	<i>p</i> Value	<i>p</i> <sub>het</sub> <sup>b</sup>
rs2243100	17	4,779,777	SCL25A11	Locus-region	C/T	0.20	0.011	0.006, 0.017	5.00 × 10 <sup>-5</sup>	0.32
rs2243093	17	4,776,675	GP1BA	5' UTR	T/C	0.22	0.012	0.007, 0.017	5.94 × 10 <sup>-5</sup>	0.42
rs2660896	12	94,947,913	LTA4H	Intron	C/T	0.25	-0.010	-0.015, -0.005	7.94 × 10 <sup>-5</sup>	0.96
rs2242406	16	74,131,531	CHST5	Intron	C/T	0.01	-0.034	-0.051, -0.017	9.46 × 10 <sup>-5</sup>	0.18

<sup>a</sup> MAF = minor allele frequency.

<sup>b</sup> *p*-for-heterogeneity by Cochran's Q.



**Fig. 4.** This plot shows the association of rs290481 with ABI for each of the discovery and replication studies, and then results with these studies combined by meta-analysis. The x-axis contains beta coefficients for the association of rs290481 with ABI in the discovery and replication studies, and the y-axis shows studies or groups of studies. Circles represent the beta coefficient (except for overall replication and discovery + replication meta-analysis results, where beta coefficients are designated by a diamond), and error bars are 95% confidence intervals. *p*-Values for heterogeneity are by Cochran's Q. Study abbreviations are as follows: ARIC = atherosclerosis risk in communities, CFS = Cleveland family study, CHS = cardiovascular health study, FHS = Framingham heart study, MESA = multi-ethnic study of atherosclerosis, KORA F3 and KORA F4 = cooperative research in the region of Augsburg, PDHS = Penn diabetes heart study, GeneSTAR = genetic study of aspirin responsiveness, PREVEND = prevention of renal and vascular end-stage disease, Copenhagen = Copenhagen city heart study, NHANES = national health and nutrition examination survey, LIPAD = Linz peripheral arterial disease, CAVASIC = cardiovascular disease in intermittent claudication, and GenePAD = genetic determinants of peripheral arterial disease.

**Table 3B**  
Candidate gene SNP associations for PAD (ABI < 0.9): discovery meta-analysis, *p* < 10<sup>-4</sup>. African Americans, *n* = 7267.

SNP	Chr	Physical position	Nearest gene	Feature	Major/minor allele	MAF <sup>a</sup>	Odds ratio	95% CI	<i>p</i> Value	<i>p</i> <sub>het</sub> <sup>b</sup>
rs4987756	18	59,060,091	<i>BCL2</i>	Intron	A/G	0.01	2.99	1.88, 4.76	3.78 × 10 <sup>-6</sup>	0.63
rs1256143	14	63,981,380	<i>MTHFD1</i>	Intron	C/T	0.19	1.72	1.21, 1.66	1.43 × 10 <sup>-5</sup>	0.05
rs13004470	2	242,159,756	<i>BOK</i>	Intron	C/T	0.18	1.39	1.19, 1.62	4.69 × 10 <sup>-5</sup>	0.81
rs9830448	3	154,349,978	<i>RAPB2</i>	Locus region	C/A	0.07	1.58	1.28, 2.02	4.79 × 10 <sup>-5</sup>	0.73

<sup>a</sup> MAF = minor allele frequency.  
<sup>b</sup> *p*-for-heterogeneity by Cochran's Q.

genetic risk factor for susceptibility to type 2 diabetes [22–25] and *CYP2B6* affects smoking behavior [26] and thus may be important in tobacco-related diseases such as PAD. However, we were unable to replicate the SNP–ABI associations in additional samples from population-based studies or clinically based samples. Furthermore, the associations were not detected in African Americans. We did not observe any significant associations for ABI or PAD in African Americans, possibly due to the relatively small sample size limiting our power to detect associations.

#### 4.1. In the context of the current literature

Genetic factors leading to susceptibility to PAD remain largely unknown but are likely to be attributed to variants in many genes, each with small effects [13] or possibly from rare variants (minor allele frequency < 1%) with larger effects. While many of these variants may lead to risk for PAD through effects on established risk factors or shared effects with CAD and other atherosclerotic diseases [27], other variants may uniquely influence development



of arterial disease in the lower extremities. Although our findings after the discovery stage did not bear out in the replication samples, the two genes are interesting candidates for ABI in light of the current literature and deserve some discussion. Genome-wide association studies of individuals of European ancestry have consistently reported an association between genetic variants of *TCF7L2* and type 2 diabetes that has been confirmed in Japanese and African American samples [19,22–24,28–30]. However, our SNP in *TCF7L2*, rs290481 was not significantly associated with type 2 diabetes in large scale association analysis [21]. The replicated index SNP in *TCF7L2* associated with type 2 diabetes is rs7902146. Further, rs290481 is not in linkage disequilibrium with rs7902146 ( $r^2 = 0.001$ ) [21]. *TCF7L2* encodes a high mobility group (HMG) box-containing transcription factor that is involved in the Wnt signaling pathway [31] and is associated with impaired beta cell function, impaired insulin secretion and increased hepatic glucose production. Therefore, the *TCF7L2* association in our discovery cohorts that presented even after adjusting for type 2 diabetes might deserve further attention in functional studies to elucidate its role in atherosclerosis.

The association between *SYTL3* and ABI may be mediated by lipoprotein(a) (Lp(a)). A genome-wide association study in a small founder population of 386 Hutterites identified an association between the extended *LPA* gene region on chromosome 6q26–q27 including *SYTL3* SNPs with Lp(a) levels [32]. Genetic variation within the *LPA* gene region including a very common copy number variation and other genetic variants explain up to 90% of Lp(a) concentrations [33]. Since Lp(a) concentrations and genetic variants within the *LPA* region are a strong risk factor for cardiovascular disease [34], it might well be that a SNP in the *SYTL3* gene reflects a signal from *LPA*. Polymorphisms within the *LPA* gene region were associated with PAD in a past study [35]. Lp(a) may be an independent risk factor for PAD [36] but results are conflicting [37,38].

#### 4.2. Strengths and limitations

To our knowledge this study is the largest candidate gene association study concerning ABI conducted in both individuals of European ancestry and African Americans and includes the most extensive number of candidate genes investigated. In the CARE consortium, imputed GWAS data is available on the African-American participants; however, given our relatively modest African-American sample size, we chose to perform analysis of the IBC chip first. We have also chosen the IBC chip because it was specifically designed as a large scale cardiovascular-centric candidate gene array, and the genetic variants on the chip were informed by GWAS for vascular and inflammatory diseases as well as expression QTLs for atherosclerosis.

Several limitations of our candidate gene meta-analysis merit comment: (i) The ankle-brachial blood pressure measurement protocols used in the studies were heterogeneous. Hence, phenotype heterogeneity may have impacted our ability to detect associations. (ii) The ARIC study contributed over 40% of the European ancestry sample and measured ABI in only one leg which may have led to phenotype misclassification most problematic for the PAD phenotype. The mean ABI did differ significantly between European Americans in ARIC and European Americans from the other CARE cohorts (all  $p < 0.05$ ) with mean  $\pm$  SD of the ARIC ABI  $1.12 \pm 0.13$ , CHS ABI  $1.06 \pm 0.15$ , CFS ABI  $1.08 \pm 0.10$ , FHS ABI  $1.13 \pm 0.12$ , and MESA ABI  $1.11 \pm 0.12$ . However, a sensitivity analysis excluding the ARIC samples showed parameter estimates of similar size. (iii) Not all studies had information on lower extremity revascularization, which may also have contributed to PAD misclassification. In general, these misclassifications should cause bias toward the null. (iv) Control selection bias could have affected our PAD results in

some way, although given that all of our studies except one (the PDHS) contributing to the PAD analysis were prospective cohort studies where knowledge of PAD would not affect exposure (i.e. genotype status) and the genotype precedes prevalent PAD, this is of lesser concern. For ABI analyses, we also analyzed our clinical replication samples separately by case-control status to avoid additional bias or heterogeneity. (v) Although we adjusted for population stratification using principal components in the European ancestry analysis and global ancestry in the African-American analysis, residual confounding could still be present. (vi) Our sample of African Americans was modest in size and likely limited our power to detect associations. For example, given the sample size of African-Americans we included and a risk allele frequency of 0.10, we only had 80% power to detect an increment in ABI of approximately 0.02 or greater per each copy of the risk allele. The observed effect size of the experiment-wide significant SNPs in European ancestry participants was much smaller than this value. According to our calculations in the methods section, for the European ancestry analyses, we can detect modest differences in ABI (similar to the ones we observed in this study), but are likely underpowered for PAD.

Some of the mentioned limitations might have contributed to the observation that the most important findings from the discovery phase could not be confirmed in the replication phase. However, it is unlikely that this fully explains the differences between the two study stages which necessitate additional large study samples.

## 5. Conclusions

The search for genes influencing ABI and PAD remains challenging. Although we cannot claim new findings in our study, two associations at the discovery stage for ABI (*SYTL3*, *TCF7L2*) may deserve further attention in other populations and functional studies. Further study of the genes identified in this study for ABI (*SYTL3*, *TCF7L2*) and PAD (*CYP2B6*) is warranted in other populations as further investigation of the function of these loci may uncover important biological insights into the pathogenesis of PAD. Identification of main effects may have been difficult in our study due to the presence of interactions and heterogeneity across participating studies. New and more powerful approaches to PAD gene discovery are sorely needed.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2012.01.039.

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